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In vitro anti-inflammatory and acetylcholinesterase inhibition efficiency of plant extracts from Sinai-Egypt

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Ten plant extracts were prepared and tested in *in vitro* assays against COX-2, COX-1 and acetylcholinesterase with evaluation of their antioxidant properties. The tested extracts exhibited varied anti COX-2 effect and they were superior to celecoxibe (inhibition percentage was 42.67% at 50 µg/mL), reference drug. *Lavandula coronopifolia* and *Scrophularia Libanotica* extracts were the efficient inhibitors (100% and 91% at 50 µg/mL respectively). *Launaeaspinosa* and *Pulicaria undulata* were the powerful AChE inhibitor (IC_{50} values were 16.69 and 29.06 µg/mL, respectively) followed with *L. coronopifolia* and *S.libanotica* extracts (IC_{50} values were 61.89 and 49.83 µg/mL, respectively) and they were efficient in scavenging superoxide radicals and metal ions, nitric oxide formation inhibition, as well as, lipid peroxide production suppression. *L. coronopifolia* and *S. Libanotica* extracts can be introduced as natural cyclooxygenase-2 inhibitors without affecting cyclooxygenase-1 whereas *L. spinosa* and *P. undulata* extracts were potent suppressor for AChE with robust antioxidant properties which suggest the possibility of using the four extracts, *L. coronopifolia*, *S. libanotica*, *L. spinosa* and *P. undulata* as natural agent in treating neurodegenerative disorder.

Keywords: Anti-inflammatory, Acetylcholinesterase, Cyclooxygenase inhibition, Sinai Egyptian wild plants

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Saint Catherine's Reserve, an ecological and cultural heritage site, is the Sinai Peninsula's most biologically diverse region[1,2]. Since ancient Egyptian times, herbal plants have been used as a source of medications which have been confirmed by several studies through isolation and identification of biologically active metabolites as pharmaceutical drugs[3-6].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly utilized pharmaceutical agents in many diseases. NSAIDs mechanism of action is contributed to their ability to inhibit prostaglandin biosynthesis, the activity possessed by two individual cyclooxygenase isozymes, COX-1 and COX-2. Researchers around the world work for discovery of new potent naturally NSAIDs selective for COX-2, with fewer side effects than traditional NSAIDs[7]. The arachidonic acid (AA) is enzymatically transforms to endoperoxide-containing prostaglandin G2 (PGG2), potent bioactive lipid messengers derived from AA, in the cyclooxygenase reaction. The reduction of a hydroperoxyl to a hydroxyl form in PGG2 and PGH2, respectively, via a separate peroxidase enzyme to produce Isomerases and oxidoreductases bioactive produces using PGH2 as substrate. Therefore, discovery of selective COX inhibitor is major important way to stop this inflammatory cascade[8]. Natural products have been one of the most successful sources of potential drug leads and continue to provide unique structural variety. In addition to the natural products drugs used in pharmaceutical application, it can be used as templates for synthesis and semi-synthesis of novel drugs for treating humankind's diseases[9]. Medicinal plants have been used for thousands of years as traditional treatments and natural products (NPs) from these plants are still the basis for most modern medicines[10-12]. Sinai Peninsula is one of the significant places of medicinal plants in Egypt, a unique linkage features between Africa and Asia, were observed to have wide range of traditional usage for humans or grazing for animals. The Sinai medicinal plants distribution, utilization in folk medicine including active constituents continue to attract the attention of many ecologists, taxonomists and phytochemists[1,13-15]. The current study include the

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selection of Sinai wild plant species based on the unique ecosystem giving rise to great plant diversity due to large variations in landforms, water resources, aridity conditions and temperatures. Herein, we aimed to give a scientific approval and/or new biological finding for some of the wild plants growing in Sinai Peninsula.As a part of our continuous research for natural products discovery from plants growing in Egyptthrough evaluation of ten plant extracts as anti-inflammatory natural products by evaluating their cyclooxygenases inhibitor activity and testing their effect against acetylcholinesterase which play important role in many neuroinflammatory diseases[16-21].

Materials and methods

Plant material and extraction

The air-dried aerial parts of wild plants "*Launaea spinosa* (SK-219), *Teucrium polium* (SK-105), *Tanacetum sinaicum* (SK-120), *Pulicaria undulate* (SK-103), *Chiliadenus montanus* (SK-1001), *Ballota undulata* (SK-113), *Scrophularia libanotica* (SK-107), *Euphorbia sanctae-catharinae* (SK-212), *Lavandula coronopifolia* (SK-215) and then *Stachys aegyptiaca* (SK-213)*"* were collected in June 2014 from South Sinai, Egypt. The identified and voucher specimens have been deposited in the herbarium of Saint Catherine protectorate Egypt. The collection was completed under the agreement of Saint Catherine protectorate for scientific reasonsthrough officially permission from the National Research Center. The powdered aerial parts of each plant (100 g) were extracted at room temperature with CH_2Cl_2-MeOH (1:1). The crude extracts were achieved using rotary evaporator concentrated filtrate solvents extract, resulting in 10 crude extracts containing various compounds polarity.

Chemicals

1,3-diethyl thiobarbituric acid (DETBA), Ammonium thiocyanate and linoleic acid were purchased from E. Merck. Peroxidase, phenazine methosulphate (PMS)-nicotinamide adenine dinucleotide (NADH), Cyclooxygenase enzyme (COX-1 from sheep, EC. 1.14.99.1 or COX-2), 2, 2-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid) diammonium salt, nitroblue tetrazolium (NBT), Tris-HCl buffer, Greissreagent, sodium nitroprusside, ferrous chloride, 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), nicotinamide adenine dinucleotide (NADH), Ascorbic acid (Vc), butylated hydroxytoluene (BHT), Leuco-2,7-dichlorofluorescien diacetate, hematin, arachidonic acid, Tris-buffer, acetylcholinesterase (E.C. No. 3.1.1.7) Type VI-S: from Electric Eel, serine hemisulfate salt, acetylthiocholine iodide, DTNB (dithiobis nitrobenzoic acid), were purchased from Sigma-Aldrich, USA.

Cyclooxygenases inhibitory activity

The optimum method designated by Larsen *et al*. (1996)[22] is endorsed for the determination of cyclooxygenase inhibitors activity of natural or synthetic materials. The leuco-dichlorofluorescein (L-DCF) oxidation by the hydroperoxide formed in the cyclooxygenase reaction in the presence of phenol can be used as a sensitive spectrophotometric assay for PGH synthase activity[22] (Larsen *et al*., 1996). The concentrations 25, 50, 100, 200 and 400 µg/mL for fractions were investigated and Celecoxib was used as a reference compound.

Acetylcholinesterase inhibition assay

Ingkaninan *et al*. (2003)[23] method is recommended for determination of acetylcholinesterase enzymatic activity. The reaction mixture contains AChI (15 µM), DTNB (3 µM) and Tris–HCl buffer (50 µM, pH 8) dissolved in ethanol then each sample, plant extracts at 25, 50, 100, 200 and 400 µg mL-1, was added and accompanied with blank sample. One ml of reaction mixture and tested extracts at different concentration in three replicates were transferred to cuvette. The enzyme solution (0.28 U mL−1 25 µL) were exchanged by the same volume of the buffer in the reaction cuvette. The absorbance was examined for 5 min at 405 nm for enzymatic activity calculation[23] and Eserinehemisulphate was used as a reference drug.

Nitric Oxide radical scavenging activity

Plant extracts at 25, 50, 100, 200 and 400 µg/mL were tested using Greiss reagent[24]. The reaction mixture (2 mL) including various extract concentrations as well as standard compounds and SNP (10 µM) were incubated at 25ºC for 150 min in phosphate buffered saline (PBS) pH 7.4. After incubation, the reaction mixtures for each tested sample (1 mL) were diluted with Greiss reagent (1:1, v/v) and the absorbance was measured at 540 nm.

Antioxidant capacity

The total antioxidant capacity of tested extracts at 25, 50, 100, 200 and 400 µg/mL were determined using Peroxidase-ABTS technique[25,26]. The mixture was kept in dark for one hour and then one mL of each sample or standard was added followed monitoring of absorbance at 734 nm using the following equation for total capacity calculation:

Total antioxidant activity (%) = [1- (A sample/A control)] x 100.

Metal chelating activity

The chelating of ferrous ions by extracts and standards was estimated by the method of Dinis *et al*.,[27]. Briefly, extracts and standards of various tested concentrations were added to $FeCl_2$ (2 µM). Ferrrozine was used for reaction initiation (5 µM) and the mixture was shaken vigorously and left standing at room temperature for ten minutes. The absorbance was then measured at 562 nm in a spectrophotometer. The inhibition percentage of ferrozine-Fe^{2+} complex formation was given by the formula: Inhibition %=[(A0-A1)/ A0]×100, where A0 was the absorbance of the control and A1 was the absorbance in the presence of the sample of extracts and standards. The control contains $FeCl_2$ and ferrozine.

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of extracts was based on the method described by Liu *et al*.[28]. Superoxide radicals are generated in phenazine methosulphate (PMS)–nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). L-ascorbic acid and BHT were used as controls. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula: Inhibition %=[(A0-A1)/A0]×100, where A0 was the absorbance of the control (l-Ascorbic acid), and A1 was the absorbance of extracts or standards.

Lipid Peroxidation- Ammonium Thiocyanate Method

The antioxidant activity of plant extracts under study was investigated using a modified Gulcin method[29]. A pre-emulsion and samples preparation were performed as previously described by us[30]. The percentage of lipid peroxidation inhibition was calculated according to the following equation: Inhibition% = [(A0-A1)/A0] x 100 (1), Where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of tested extracts and/or standard compounds.

Statistical analysis

All data are expressed as means of triplicates±S.D. One- way ANOVA was used for analysis of variance for statistical evaluation followed with Tukey's Multiple Comparison test using SPSS 9 program.

Results and Discussion

The inhibitory effect of plant extracts against COX-2 and COX-1 were evaluated as indicator of their role in inflammatory response in comparing to celecoxib, reference compound. Data recorded indicated the gradual increasing inhibitory effect of tested extracts with increasing concentrations and they exhibited more inhibition percentages at (50, 100 and 200 µg mL-1) than celecoxibe except *B. undulata* and *C. montanus* extracts, Fig. (1). *L. coronopifolia* presented significant inhibition ($p \leq 0.05$) and it was powerful in COX-2 inhibition (100% at 50, 100 and 200 µg mL-1) while the minimum mean inhibitory value was observed with *B. undulata*, extract (74.34% at 200 µg mL-1). *S. libanotica* extract completely inhibited COX-2 at 100 and 200 µg mL-1 whereas *S. aegyptiaca*, *E. sanctae-catharinae*, *L. spinosa*, *T. sinaicum* and *P. undulata* extracts showed the same effect at the highest concentration only.

On the other hand, the plant extracts showed varied effects to COX-1. Generally, they partially inhibited COX-1 with low values comparing to reference drug, celecoxib, at all concentrations in a concentration dependent manner. The minimum inhibitory effect

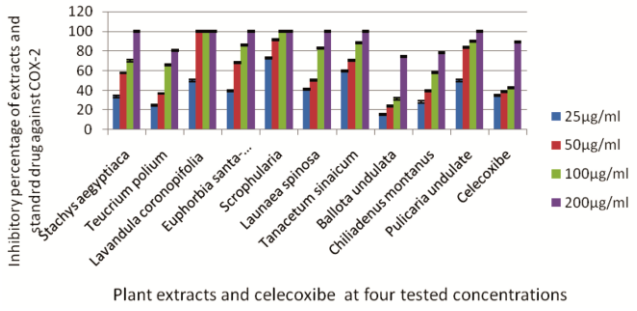

Fig. 1 — Inhibitory effect of plant extracts and standard drug against COX-2. Data are expressed as mean of triplicates ± S.D. Significant difference calculated as compared to celecoxibe ($p<0.05$).

was recorded for *T. polium*, *L. spinosa* and *T. sinaicum* (15, 14 and 17%, respectively at 200 µg mL-1) whereas *L. coronopifolia*, *S. libanotica*, *B. undulata*, *C. montanus* and *P. undulata* produced the same effect (24-26% at 200 µg mL-1), Fig. (2). *S. aegyptiaca* and *E. sanctae catherine* extracts showed the highest inhibition values (30% at 200 µg mL-1) which were lower than celecoxibe (61%).

Itis evident from cyclooxygenases presented results that the ten tested extracts significantly inhibited COX-2 with low inhibitory effect against COX-1 as compared to the reference drug. *L. coronopifolia* extract exhibited the highest inhibition percentage (100% at low conc., 50 µg mL-1) against COX-2 with reduced COX-1 inhibition (20.71%) at the same concentration while celecoxibe reached its maximum inhibitory COX-2 values (89%) at 200 µg/mL with

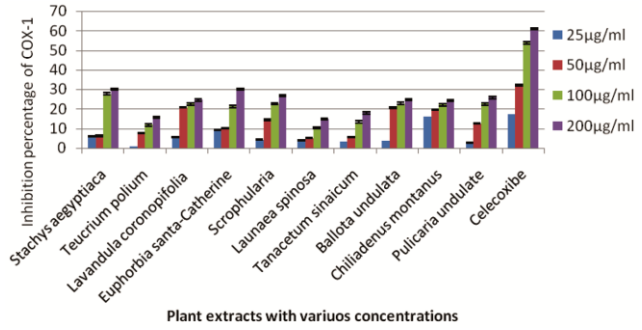

Fig. 2 — Inhibitory effect of plant extracts and standard drug against COX-1. Data are expressed as mean of triplicates ± S.D. Significant difference calculated as compared to celecoxibe ($p<0.05$).

61% for COX-1 which refers to the safety effect *of L. Coronopifolia* extract to incorporated in inflammatory diseases with keeping the cell maintenance. The plant extracts were ascending arranged as COX-1 suppressor in the following order; *L. spinosa*, *T. polium*, *T. sinaicum*, *P. undulata*, *C. Montanus* and *B.undulata*, *S. libanotica*, *E. sanctae-catharinae*, *L. coronopifolia* and then *S. aegyptiaca*.

Neurodegenerative diseases have multi risk factors therefore the treatments always include many pathways. In this concern, it is preferable to use drugs that have many effects incorporated in different pathway mechanisms. Through this point of view, ten plant extracts were investigated for their acetylcholinesterase inhibitory effect in different concentrations. Increasing the extracts concentration produced significant increments in acetylcholinesterase inhibition percentage; most of plant extracts completely inhibited acetyl-cholinesterase at 200 µg/mL. Despite *L. spinosa* occupied the sixth inhibitory level with COX-2, it showed remarkable inhibitory effect against acetyl-cholinesterase (IC_{50} was 16.69 µg/mL), Table 1**.**

P. undulata and *S. aegyptiaca* extracts came in the second activity level with IC_{50} values at (IC_{50} were 29.06 and 30.84 µg/mL, respectively). However, *T. sinaicum* and *S. libanotica* presented the same IC_{50} values (49 µg/mL) followed with *L. Coronopifolia* (61.89 µg/mL) then *B. undulata*, and *E. sanctae-catharinae*(74.56 and 79.25 µg/mL, respectively), while *T. polium* showed the lowest activity level with IC_{50} value 135.50 µg/mL.

Table 1 — Inhibitory effect of plant extracts and standard compound against Acetylcholinesterase

Plant extract and standard	Tested concentrations (µg/mL)				IC_{50} (µg/mL)
	25	50	100	200	
Stachys aegyptiaca	18.18±0.12^{a}	65.52±0.42	77.76±0.91^{h}	81.12±0.36^{k}	30.84
Teucrium polium	19.26±0.22^{a}	19.44±0.41	38.44±0.82	61.70±0.39	135.50
Lavandula coronopifolia	30±0.34^{c}	36±0.33	100±0.61	100.00±0.27^{j}	61.89
Euphorbia sanctae-catharinae	36.66±0.27^{b}	42.94±0.27^{e}	78.29±0.9^{h}	100.00±0.41^{j}	79.25
Scrophularia libanotica	48.22±0.09^{d}	76.49±0.19^{f}	78.29±0.72^{h}	100.00±0.46^{j}	49.83
Launaea spinosa	78.64±0.31	78±0.36^{f}	84.43±0.68^{i}	100.00±0.46^{j}	16.69
Tanacetum sinaicum	22.81±0.26	48.78±0.64^{g}	84.85±0.31^{i}	100.00±0.71^{j}	49.73
Ballota undulata	36.42±0.60^{b}	48.72±0.44^{g}	79.35±0.42^{h}	100.00±0.81^{j}	74.56
Chiliadenus montanus	31.22±0.41^{c}	42.18±0.38^{e}	63.72±0.19	83.46±0.61^{k}	69.76
Pulicaria undulata	49.26±0.35^{d}	74.51±0.09^{f}	93.28±0.32	100.00±0.37^{j}	29.06
Eserinehemisulphate (positive control)	It reproduced IC_{50} = 0.03 µg/mL				

Data are presented as mean of triplicates ± standard deviation. Data were analyzed by ANOVA one way followed with Post Hoc for multiple comparisons. Groups have the same letter have no significant difference between them. IC_{50} is a concentration that reproduces 50% inhibition.

The antioxidant compounds participate strongly in the earlier stage of Alzheimer by many means including clearance of toxic materials from cells that can affect biomolecules[31]. Nitric oxide, small toxic bioproduct in human cells, formation plays an important role in cyclooxygenase-2 activity induction, which activates the inflammatory cascade, therefore, the inflammation related diseases; cancers and neurodegenerative diseases, inhibition of nitric oxide formation is an important factor in cyclooxygenase-2 inhibition in human biological system[32]. The tentestedextracts significantly prevented nitrite formation by quenching the nitric oxide. The IC_{50} values ranged from 11 µg mL^{-1} to 19 µg/mL. *B. undulata*, *C. montanus* and *P. undulata* produced the same nitrite formation inhibition level and they were the most effective extracts, IC_{50} values were (11.53, 12.63 and 11.44 µg/mL, respectively) followed with *S. aegyptiaca*, *E. sanctae-catharinae* and *L. spinosa* (13.31, 13.53 and 14.87 µg/mL, respectively). However, *T. polium*, *S. libanotica* and *T. sinaicum* represented the languorous effect which is nearly the same standard materials, Table 2**.**

Tested plant extracts quenched superoxide radicals produced by phenazine in IC_{50} range from 27 to 51 µg mL-1. *S. aegyptiaca, S. libanotica* and *T. sinaicum* extracts (IC_{50} values were 36.95, 36.22 and 35.46 µg/mL, respectively) came after *L. spinosa*, *C. montanus* and *P. undulata* which extensively trapped superoxide radicals in the reaction media, IC_{50} values were 28.29, 29.56 and 27.43 µg/mL, respectively. Nevertheless, *T. polium*, *L. Coronopifolia*, *E. sanctae-catharinae, B. undulate* proceeded the lowest effects, IC_{50} were 43.64, 43.25, 41.71 and 43.26 µg/mL, respectively (Table 2). The presence of excessive metal ions in tissue caused deteriorative effect represented in maximizing oxidative stress that contributes in many diseases[30]. *S. aegyptiaca* seems to be the potent extract in chelation of metal ion (IC_{50}, 27.31 µg/mL) followed with *L. Coronopifolia* (IC_{50}, 30.1 µg/mL), *T. polium* (IC_{50}, 39.54 µg/mL), *T. sinaicum* (IC_{50}, 42.16 µg/mL) and then *B. undulata* (IC_{50}, 39.52 µg/mL). The other extracts produced weak chelating effect against ferrous ions, *S. libanotica* (48.63 µg/mL), *E. sanctae-catharinae* and *L. spinosa* (58 µg/mL) and then *C. montanus* and *P. undulata* (61.72 µg/mL), Table 2**.** Total antioxidant capacity was determined for all extracts at four concentrations. *B. undulata* and *L. spinosa* exhibited the same IC_{50} value (18 µg/mL) followed with *E. sanctae-catharinae, T. Sinaicum* and *S. libanotica* (20 µg/mL), *S. aegyptiaca* and *C. montanus* (26 µg/mL) and then *T. polium* and *L. coronopifolia* (29 µg/mL) while *P. undulata* possessed the lowest capacity, IC_{50} value is 30 µg/mL.

Lipid peroxides production in linoleic assay was significantly suppressed by the presence of *E. sanctae-catharinae*, IC_{50} was 9 µg/mL, whereas *T. polium*, *L. coronopifolia* and *B. undulata* came at the second inhibition level, IC_{50} was 10 µg/mL, with in significant difference with *S. aegyptiaca* (IC_{50} was 11 µg/mL). On the other hand, *T. sinaicum*

Table 2 — Antioxidant properties of extracts and standards as IC_{50}values

Test Plant extract	IC_{50} (µg/mL)				
	NO scavenging	Superoxide anion scavenging	Chelation of ferrous ions	Total antioxidant capacity	Inhibition of lipid peroxidation %
Stachys aegyptiaca	13.31±1.02[a]	36.95±1.14[d]	27.31±1.16	26.12±1.33[k]	11.99±0.94[m]
Teucrium polium	19.07±0.96[c]	43.64±1.20[e]	39.54±0.97[h]	29.73±1.05[i]	10.31±1.01[p]
Lavandula coronopifolia	16.91±0.88	43.25±0.95[e]	30.10±1.13	29.52±1.11[i]	10.81±0.76[p]
Euphorbia sanctae-catharinae	13.35±1.00[a]	41.71±1.03[e]	58.45±1.14[g]	20.21±0.86[l]	9.76±0.89
Scrophularia libanotica	18.02±0.97[c]	36.22±0.95[d]	48.63±1.08	20.29±0.94[l]	13.37±1.00[m]
Launaea spinosa	14.87±1.03[a]	28.29±1.05[f]	58.61±1.00[g]	18.66±1.12[l]	13.66±1.12[m]
Tanacetum sinaicum	18.26±1.10[c]	35.46±1.10[d]	42.16±0.96	20.51±1.03[l]	12.66±1.01[m]
Ballota undulata	11.53±0.89[b]	43.26±1.22[e]	39.52±0.97[h]	18.46±0.99[l]	10.69±0.93[p]
Chiliadenus montanus	12.63±0.93[b]	29.56±1.03[f]	61.52±1.11[g]	26.12±0.85[k]	12.56±0.98[m]
Pulicaria undulata	11.44±1.04[b]	27.43±0.99[f]	61.72±1.07[g]	30.43±1.01[i]	13.36±1.03[m]
Vitamin C	17.94±0.91[c]	51.8±0.91	68.76±0.94	30.48±1.23[i]	12.34±1.14[m]
BHT	17.75±1.03[c]	43.83±0.87[e]	58.91±1.10	28.35±0.98[k]	13.25±1.02[m]

Data are expressed as mean of triplicates ± SD The significant difference evaluated $p<0.05$. There is insignificant difference between groups have the same letter. IC_{50} values are the concentration that produced 50% effect percentage.

and *C. montanus* depressed 50% of lipid peroxides formation at IC_{50} value at 12 µg/mL. The existence of 50% of lipid peroxides was blocked by extracts of *S. libanotica*, *L. spinosa* and *P. undulata* at 13 µg/mL.

Focusing on the mentioned data, it can be concluded that *L. coronopifolia*, *S.libanotica*, *E. sanctae-catharinae* and *P. undulata* extracts were effective in suppression of cyclooxygenase-2 with weak effects on cyclooxygenase-1, which support their sufficient and safe role as non-steroidal anti-inflammatory agent. The same extractscame at the second level after *L. Spinosa* and *P. undulata* as potent anti-acetylcholinesterase agent which can keep the neurotransmitters, acetylcholine, from hydrolysis with valuable effect as ion chelator, scavenger for radicals and preventer for nitric oxide formation. According to the tested activities, these extracts can be recommended for further in-vivo studies for neurodegenerative diseases.

Conclusion

The present work introduced *L. coronopifolia* and *S. libanotica* extracts as potent natural cyclooxygenase-2 inhibitors with minor effects on cell maintenance via reduced effect on cyclooxygenase-1. Additionally, their suppressive effect on acetylcholinesterase with plausible antioxidant properties which suggest the possibility of using them as natural agent in treating neurodegenerative disorder. The analgesic and anti-inflammatory activities have also been reported from other *Lavandula* species extracts including polyphenolic fractions and essential oils[33-35]. In addition, previous studies showed that the aqueous alcohol extract of *S. libanotica* showed potent anti-inflammatory activity due to its glycoterpenoids content[36, 37].

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